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DEVELOPMENT AND DEPLOYMENT OF SPECIALIZED MICRO-ORGANISMS FOR IN SITU REMEDIATION OF AQUIFERS CONTAMINATED WITH VOLATILE ORGANIC COMPOUNDS

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13. ABSTRACT (Maximum 200 words)

The objective of Phase I work was to develop and test several specialized degradative microorganisms for remediation of chlorinated solvent-contaminated aquifers. The organisms constitutively degrade chlorinated solvents, and therefore, do not require the presence of toxic co-substrates to stimulate degradation. Constitutive toluene monooxygenase (TMO) variants of P. mendocina KR-1 and several other strains were developed by first treating the cells with mutagenizing agents, then selecting variants that constitutively produced TMO. A constitutive TOM variant of P. cepacia G4 was also tested for TCE degradation. These microorganisms were also selected for adhesion-deficient variants, and thus able to penetrate aquifer sediments. Methods were developed for culturing the organisms to extremely high cell density, to reduce treatment costs, and so that they contain elevated levels of storage polymers. The storage polymers stimulate and prolong their activity to degrade more TCE in situ. The goal of the proposed Phase II work is to expand upon the success of Phase I work by demonstrating the utility of our specialized degradative microorganisms to remediate contaminated aquifers.

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PREFACE

This report summarizes research activities conducted by Envirogen, Inc. for Armstrong Laboratories through U.S. Department of Defense Small Business Innovative Research (SBIR) contract no.F08637 94 C6016 "Development and Deployment of Specialized Microorganisms for *In Situ* Remediation of Aquifers Contaminated with Volatile Organic Compounds" during the performance period May 12, 1994 through December 12, 1994.

The work summarized in this report have been conducted by Robert J. Steffan (Research Manager, Bioremediation Technologies), Marina Tugusheva, Charles C. Condee, Simon Vainberg, Sheryl Streger, Jennifer Gaudet, Kevin McClay (Research Associates) and Mary DeFlaun (Applications Manager, Bioremediation Technologies) of Envirogen, Inc. William Guarini (Vise President, Government Programs) of ENVIROGEN served as the business and commercialization representative, and Allison Thomas of Armstrong Laboratory was the Project Officer.

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EXECUTIVE SUMMARY

A. OBJECTIVE

The goal of this Phase I SBIR project was to develop specialized bacteria for the *in situ* destruction of chlorinated solvents in groundwater. The specific objectives of the project were:

1. develop bacteria that could constitutively degrade chlorinated solvents;
2. select adhesion deficient variants of constitutive TCE-degrading bacteria;
3. determine culture conditions for high density growth and bacterial storage polymer production by the strains developed in task 1 and 2;
4. determine the ability of the selected strains to degrade TCE in groundwater and in the presence of TCE-contaminated aquifer material;
5. determine the effects of bacterial storage polymers on TCE degradation;
6. measure migration of the selected strains through aquifer materials; and
7. use the data collected to generate preliminary cost estimates for the technology.

B. Background

Chlorinated solvents are a family of compounds including trichloroethylene (TCE), dichloroethylene (DCE), tri-, and dichloroethane (TCA and DCA) and a number of chemically-related compounds. These compounds have been used widely in industrial and other applications, and are major contaminants throughout the United States. Of these compounds, TCE has become the model target compound because of its ubiquity at contaminated sites.

Although many organisms that degrade TCE have been identified, no known organism is capable of using TCE as a sole source of carbon and energy. Biological attack is co-metabolic, thus, biological destruction of TCE requires the presence of a co-substrate that acts both a food source and an inducer to maintain enzymatic activity. Unfortunately, the co-substrate is generally a toxic compound (e.g. toluene or phenol). This property, along with its chemical stability, has led to the recalcitrance of TCE in the environment.

ENVIROGEN has used an integrated approach to develop specialized microorganisms for *in situ* remediation of chlorinated solvents. The organisms developed maintain expression of their degradative genes in the absence of inducing chemicals (constitutive), have reduced adhesion properties (adhesion deficient), and can be grown to maximize their energy storage material (energy enriched) to prolong degradative activity *in situ*. Their development has not required the use of genetic engineering to achieve the project goals, but rather involved the use of classical mutagenesis and selection techniques. The organisms have been tested for their ability to remove TCE and related chlorinated solvents from contaminated aquifer material, to penetrate aquifer sediments, and to efficiently utilize energy reserves to prolong degradative activity and minimize oxygen burdens.

ENVIROGEN's technology will have distinct advantages over competitive processes because of the several unique developments in biocatalyst design and application methods. Because of the expected effectiveness of the *in situ* technology, ENVIROGEN believes that it will be applicable to remediation of many chlorinated solvent-contaminated sites located nationwide.

C. SCOPE

This report encompasses work conducted by ENVIROGEN during the period of May 12, 1994 through December 12, 1994.

D. METHODOLOGY

The development of constitutive TCE degrading strains involved the use of chemical mutagenesis to generate toluene minus mutants of toluene (and TCE) degrading bacteria. The toluene minus mutants were then screened for revertants that obtained a constitutive toluene degradation phenotype. Additionally, a constitutive TCE variant, *P. cepacia* PR1301c, developed by Dr. Malcolm Shields of the University of West Florida was provided as a gift for use in this study.

Adhesion deficient variants were selected by using a column enrichment technique developed by ENVIROGEN scientists. In some cases, isolation of adhesion deficient strains was aided by exposing the cells to chemical mutagens before column enrichment. Migration through aquifer materials was measured by constructing columns of aquifer solids and comparing the rate of microorganism migration to the rate of migration of a conservative tracer. High density cell growth and storage polymer production was accomplished by using a fed-batch fermentation procedure. The ability of the strains developed in this study to degrade TCE was determined by performing microcosm studies with groundwater and aquifer sediments.

E. TEST DESCRIPTION

This study involved first developing organisms that could constitutively degrade TCE. Toluene degrading strains that co-metabolically degraded TCE in the presence of toluene or phenol were selected as test organisms. Once constitutive TCE degrading variants were selected, they were subjected to enrichment for adhesion deficient variants that retained their constitutive TCE degrading phenotype. Column migration studies were then performed to test the ability of the strains to migrate through aquifer materials.

During and after the selection process fermentation protocols were performed to determine conditions required to generate high cell densities ($O.D._{550}>60$) with high intracellular concentrations of bacterial storage polymers (>50% dwt). TCE degradation studies and survival experiments were also performed to test the ability of the selected strains to degrade TCE in aquifer materials and groundwater.

After completion of much of the TCE degradation studies, preliminary cost estimates were performed to determine the economic advantages of an in situ bioaugmentation treatment system. The potential costs were calculated based on a known TCE-contaminated aquifer in New Jersey, and the cost of the technology was compared to the cost of a pump-and-treat system and an air sparging system.

F. RESULTS

During this Phase I project, ENVIROGEN has successfully developed several bacteria capable of various levels of constitutive expression of TCE-degrading genes/enzymes. The wild-type strains used for this work included *P. cepacia* G4, *Pseudomonas* sp. ENVBF1, ENV110, and ENV113. The most stable mutant tested, however, was *P. cepacia* $PR1_{301c}$ which was provided by Dr. Malcolm Shields. Experiments performed during this study demonstrated that, when properly grown, *P. cepacia* $PR1_{301c}$ will continue to degrade TCE for at least 5 times longer than wild-type *P. cepacia* G4. Because the constitutive variants continue to express TOM in the absence of inducing substrates, the cells continue to degrade TCE as long as the cells have sufficient energy reserves to catalyze the reaction.

By using our adhesion assay, we selected natural variants of *P. cepacia* G4, *M. trichosporium* OB3b, *P. cepacia* $PR1_{301c}$, *P. mendocina* KR1, and *P. cepacia* ENV BF1 that are adhesion-deficient in comparison to the wild-type. Some of the variants adhere to the sand columns at <10%, while the wild type strains adhere at >90%. Column migration studies demonstrated that several of these strains migrate through a 25 cm sand-column at the same rate as a chloride tracer.

The fermentation protocols developed during this project allowed growth of *P. cepacia* G4, $PR1_{301c}$ and *Pseudomonas* ENV BF1 to optical densities ($O.D._{550}$) of greater than 65 (80 g/L), and so that they contained as great as 60% dry weight of storage polymers. Only about 5 days were needed to generate the cells.

TCE degradation studies with the energy enriched constitutive variants demonstrated that the cells could continuously degrade TCE for up to 165 hr. Non-energy enriched constitutive TOM cells degraded TCE for only about 48 hours. These findings demonstrate that the degradative bacteria can now be cultured so that they have extended TCE degrading capabilities.

Preliminary cost estimates demonstrated that in situ bioaugmentation with the strains developed during this study can be a cost effective technology for remediating TCE-contaminated aquifers.

G. CONCLUSIONS

This study resulted in the successful development of improved bio-catalysts for in situ remediation of TCE-contaminated aquifers. The bacterial strains developed can degrade TCE in the absence of toxic inducing co-substrates, and they do not adhere to aquifer solids. They are capable of migrating through columns of aquifer material at the same rate as conservative tracer molecules, and they rapidly degrade TCE in groundwater and in the presence of TCE-contaminated aquifer sediments. The organisms can survive in aquifer microcosms for >28 days, and they actively degrade TCE for >160 hr without addition of an exogenous energy source. The fermentation methods developed during this project make the addition of degradative bacteria to aquifers economically feasible when compared to competitive technologies.

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H. RECOMMENDATIONS

The results of this project are the first step in developing a commercial bioaugmentation technology for destruction of TCE in contaminated aquifers. Follow-on work should include large-scale laboratory pilot testing and field demonstration. Pilot testing should focus on generating data needed to develop predictive models for field application. The models should then be used to design an appropriate field demonstration study to evaluate the efficacy of the technology, and to develop definitive costs estimates for field implementation

Field testing should include monitoring TCE degradation, bacteria migration and survival, cell dosing requirements, and oxygen and nutrient requirements. The field study should also evaluate methods for quantifying biological TCE degradation and determine optimum deployment system configurations.

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1. INTRODUCTION

A. Project Goals. The goal of this Phase I SBIR project was to develop specialized bacteria for the *in situ* destruction of chlorinated solvents in groundwater. The organisms developed during this project rapidly degrade chlorinated solvents such as trichloroethylene (TCE), dichloroethylene (DCE), and vinyl chloride (VC) without the requirement of toxic inducing substrates (eg. toluene and phenol). The organisms also exhibit reduced adhesion properties (adhesion deficient), thereby allowing them to penetrate further into aquifer materials than wild-type strains. Furthermore, we developed methods for culturing the organisms to extremely high cell densities, thus reducing treatment costs. The organisms, when grown to high cell densities, also contain elevated levels (to >60% dwt) of bacterial storage polymers. The storage polymers allow them to degrade greater amounts of chlorinated solvents than cells without storage polymers, further decreasing treatment costs.

B. Identification of the Problem. The economic burden to the United States created by its pollution problem is enormous. It has been estimated that greater than $340 billion dollars will be spent for Superfund and cleanup of nuclear, factory, military wastes and old tank spills in the next 20 to 30 years if we must rely on current technologies. An additional $90 billion will be spent for "corrective action" at plants still operating, including those in the oil, steel, aerospace, chemical, pharmaceutical and other industries (Kiplinger Letter, April 30, 1993). By 1997, the DOE, DOD, and private industries are expected to spend $2, $1.3, and $4.1 billion per year on remediation, respectively. An additional $1.9 and $1.6 billion/yr. will be spent on Superfund and State programs, respectively (Environmental Business Journal, Feb. 93). The Clean Air Act is expected to add an additional $1.75 to 5.5 billion/year burden for air emissions control by the late 1990s (Chemical Week, Nov. 13, 1991). In addition, it has been estimated that environmental problems in Eastern Europe, Asia, and the third world may far exceed those of the United States.

In many cases, biological approaches for remediating polluted environments provide a significant advantage over alternative technologies in both cost savings and reduction of environmental and human exposure. Biological treatment technologies can facilitate the complete destruction of hazardous chemicals without the generation of toxic emissions or by-products. Cost is reduced by lower capital expenditure and prolonged equipment life. *In situ* treatment systems provide an added advantage of destroying the contaminant in place, thereby reducing human exposures. Cost is also reduced because contaminated media does not have to be excavated, relocated, or otherwise disposed.

Biological treatment systems may also be coupled with physical extraction systems to reduce treatment costs and exposure risks. For example, ENVIROGEN recently coupled a biofilter air treatment system with a soil vapor extraction unit to successfully remediate a hydrocarbon-contaminated soil. ENVIROGEN also has coupled a vapor phase TCE bioreactor with a pump-and-treat/air stripper to remediate TCE-contaminated ground water. In each case the target contaminants were efficiently destroyed, thereby reducing total treatment costs.

ENVIROGEN and others have demonstrated that bioremediation can be an effective treatment technology for the destruction of a wide range of environmental contaminants including nitroaromatics (TNT, DNT, nitrobenzenes etc.),

hydrocarbons (BTEX, jet fuels etc.), other substituted aromatics (chlorobenzenes, aniline, styrene, acrylonitrile, etc.), poly-aromatic hydrocarbons (PAHs), chlorinated solvents (TCE, DCE, chloroform, methylene chloride, TCA, etc.), PCBs, alkanes, alkenes, hydrogen sulfide, carbon disulfide, and a wide variety of related compounds. The target compound can serve as a sole source of carbon and energy for the degradative organisms, or they may be co-metabolic substrates for which supplementary carbon sources must be present to prolong degradative activity. The extent to which compounds are destroyed is controlled by a number of factors including chemical structure and composition, concentration, solubility, hydrophobicity and environmental conditions (pH, temperature, etc.). Degradation is also controlled by the media in which the contaminant is present (air, soil, water, sludge), and the location of the contaminated media (subsurface etc.). The versatility of naturally occurring degradative microorganisms, coupled with proper system design and treatment strategies, makes it possible to greatly expand the current scope of bioremediation as a cost effective and efficient treatment alternative for remediating many of the nation's environmental pollution problems.

The Chlorinated Solvent Problem. Chlorinated solvents are a family of compounds including trichloroethylene (TCE), dichloroethylene (DCE), tri-, and dichloroethane (TCA and DCA) and a number of chemically-related compounds. These compounds have been used widely in industrial and other applications, and are major contaminants throughout the United States. Of these compounds, TCE has become the model target compound because of its ubiquity at contaminated sites.

Although many organisms that degrade TCE have been identified (Ensley, 1990), no known organism is capable of using TCE as a sole source of carbon and energy. Biological attack is co-metabolic, thus, biological destruction of TCE requires the presence of a co-substrate that acts both a food source and an inducer to maintain enzymatic activity. Unfortunately, the co-substrate is generally a toxic compound (e.g. toluene or phenol). This property, along with its chemical stability, has led to the recalcitrance of TCE in the environment.

Through funding from the Departments of Energy and Defense and internally, ENVIROGEN has performed extensive research and development of biological treatment systems for the destruction of TCE. These studies have been conducted to gain a thorough understanding of the biochemistry of TCE degradation, and to select the most appropriate degradative organisms to catalyze the reaction. The development program continued through the construction of a novel suspended growth laboratory-scale vapor-phase reactor that allowed for determining feasibility of such a reactor design. After extensive testing of the laboratory-scale reactor (Ensley and Kurisko, 1994), ENVIROGEN engineered and constructed a 4000 L pilot-scale vapor phase reactor.

The vapor phase reactor was then tested at a commercial site where it successfully degraded >95% of the TCE in a 150 to 250 µg/L TCE air stream from a ground water pump-and-treat, air stripper system. The ground water TCE concentration was approximately 4 to 7 mg/L. The reactor exceeded initial treatment estimates used to design the system. The reactor was operated for sequential 30 day test runs, and was then retro-fitted to solve problems encountered during the field demonstration. Further improvement of the reactor operating regime, based on field observations, has led to the successful continuous operation of a laboratory-scale version of the reactor for >25 weeks. The 4000 L pilot scale reactor is currently being tested under the new operating conditions at

Robins Air Force Base where it is working in combination with ENVIROGEN's pilot-scale fluidized-bed reactor to treat ground water contaminated with a mixture of JP-4 and TCE (chlorinated solvents).

C. *In Situ* Biological Treatment Technologies. In the hierarchy of bioremediation applications, it is generally accepted that direct subsurface biological treatment has distinct economic and risk advantages over above ground treatment for destroying chlorinated solvents in aquifers. For example, pump and treat technologies often require decades to achieve the desorption and mobilization of sorbed organics from aquifer and vadose zone materials. Although the pump and treat method does reduce the migration of a plume, the length of time for clean-up creates long term liability, high operating costs, and ultimately the transfer of contaminated material above ground that needs to be destroyed or taken off site for disposal.

It has thus been the thrust of researchers in this field to develop and implement direct subsurface bioremediation technologies. Within this group of technologies there is a hierarchy based upon ease of clean up and cost. The first choice has been the identification of intrinsic bioremediation whereby the natural microflora and microbial conditions exist which demonstrate that natural attenuation has already occurred and is continuing. This is always the first choice of remediation because it requires no intervention, just monitoring of the natural progress of degradation.

The second choice in the hierarchy involves the use of biostimulation of indigenous populations to remediate the target chemicals. In this case, the observation is made that a natural population exists within the contaminated zone; however, nutrients and oxygen are insufficient for microbial activity. Thus, through the introduction of an oxygen source either as hydrogen peroxide, nitrate, or air, the indigenous population can be induced to degrade the target chemicals. The lead technologies for the introduction of oxygen to the subsurface are bioventing and biosparging approaches which introduce atmospheric oxygen to the subsurface. These are often cost effective approaches for remediation utilizing the indigenous microbial population.

The third choice involves modifications of bioventing or biostimulation by which accelerated or augmented process conditions can be implemented. These include the addition of: a) vapor phase nitrogen or phosphorous; b) heating of the subsurface through radio frequency methods; and c) hydrofracturing to increase permeability and zones of influence.

In cases where intrinsic bioremediation or biostimulation do not work because of insufficient or unacclimated bacterial cultures, bioaugmentation of the subsurface can be utilized. Selected strains of bacteria with the desired catalytic capabilities (and other characteristics as described later in this proposal), can be injected directly into the contaminated zones along with any required nutrients to effect the biodegradation of the target chemicals. This technology can also be complimented through the use of RF heating and hydrofracturing. In certain cases where the natural population may be sufficient to achieve a biostimulation remediation, it still may be beneficial to add microorganisms to increase the rate of degradation and shorten the time frame for full scale remediation.

The fifth choice would be the utilization of a "funnel and gate" or "biocurtain" approach for intercepting the migration of a plume. These containment walls can be inoculated with degradative organisms or are amended to promote the growth of these bacteria. This technology is listed here as a lower priority because, although it can be a very effective containment strategy, it is in essence a subsurface pump and treat bioremediation and therefore is constrained by some of the same problems of above ground pump and treat systems. Specifically, the requirement for long operational times to effect the desorption and capture of the target chemicals.

Excellent work has been performed on these remediation strategies by researchers at the U.S. Air Force, the University of Waterloo, Stanford University, Battelle Laboratories, ECOVA Corporation, as well as ENVIROGEN. The commercial utilization of these technologies is at different levels of development with several being utilized commercially today, in particular, bioventing. Thus the choice for selection of a below ground bioremediation system is dependent on the physical, biological, and chemical characteristics that one finds at a particular site and the time frame and cost constraints presented to the owner. All of these technologies will have a place in the spectrum of bioremediation options and are either being commercialized today or will be commercialized over the next several years.

Proposed Integrated Approach to *In situ* Treatment and Anticipated Benefits

ENVIROGEN has used an integrated approach to develop specialized microorganisms for *in situ* remediation of VOCs. The organisms developed maintain expression of their degradative genes in the absence of inducing chemicals (constitutive), have reduced adhesion properties (adhesion deficient), and can be grown to maximize their energy storage material (energy enriched) to prolong degradative activity *in situ*. Their development has not required the use of genetic engineering to achieve the project goals, but rather involved the use of classical mutagenesis and selection techniques. The organisms have been tested for their ability to remove TCE and related VOCs from contaminated aquifer material, to penetrate aquifer sediments, and to efficiently utilize energy reserves to prolong degradative activity and minimize oxygen burdens.

ENVIROGEN's technology will have distinct advantages over competitive processes because of the several unique developments in biocatalyst design and application methods. Because of the expected effectiveness of the *in situ* technology, ENVIROGEN believes that it will be applicable to remediation of many VOC-contaminated sites located nationwide.

The following paragraphs discuss the specific capabilities of ENVIROGEN's biocatalyst technology.

Constitutive Variants of Degradative Microorganisms. All known natural VOC-degrading microorganisms require the presence of inducing chemicals to stimulate VOC biodegradation (Ensley, 1991). However, during Phase I of this project, ENVIROGEN has successfully developed bacteria capable of constitutive expression of VOC-degrading genes/enzymes (Table 1). Constitutive toluene monooxygenase (TMO) variants of *P. mendocina* KR-1 (ENV 307-1, ENV305-1, ENV305-2, ENV PB2b, ENV-Pb5) and several other strains were developed by first treating the cells with mutagenizing agents, then selecting variants that constitutively produced TMO. Many of the variants were extremely stable during >125 generations of

growth. Shields and Reagin (1992) first developed such mutants of *P. cepacia* G4, but those mutants were modified by transposon mutagenesis, and they may be classified as "genetically engineered". Such a classification would require their approval under the Toxic Substance Control Act (TSCA) regulations before environmental release. Dr. Malcolm Shields and colleagues at the University of West Florida have since developed a constitutive toluene *ortho*-monooxygenase (TOM) variant of *P. cepacia* G4 without the use of recombinant DNA techniques. This variant, *P. cepacia* $PR1_{301c}$, has been given to ENVIROGEN for further development, testing, and application.

Experiments at ENVIROGEN have demonstrated that, when properly grown, *P. cepacia* $PR1_{301c}$ will continue to degrade TCE for at least 5 times longer than wild-type *P. cepacia* G4. *P. cepacia* G4 degrades TCE for only about 30 hr. after which time enzyme activity ceases. (Figure 5). If additional inducing concentrations of phenol are added to the cells, TCE degradation is revived. Because the constitutive variants continue to express TOM in the absence of inducing substrates, the cells continue to degrade TCE as long as the cells have sufficient energy reserves to catalyze the reaction. ENVIROGEN scientists have demonstrated that energy enriched *P. cepacia* $PR1_{301c}$ (see below) will continue to degrade TCE for up to about 165 hours. (Figure 6). Additional degradation can be achieved if supplemental energy substrates (sugars etc.) are added.

Adhesion Deficient Variants of Degradative Microorganisms. Another factor limiting the applicability of *in situ* processes is the natural adhesive properties of native bacteria. Adhesion limits the microorganisms' penetration through soil and rock matrices (Costerton, 1992). In an *in situ* field study of *P. cepacia* G4 by Nelson et al. (1990) the data strongly suggests that movement of the microorganisms was severely retarded by the aquifer material, since non-retarded organisms would have appeared in a recovery well at about the same time after injection as the tracer, that is, within 8 to 10 hours. The hydraulic flow of the aquifer was approximately 48 feet per day but none of the injected microorganisms were observed in a monitoring well 10 feet away from the injection well until 6 days after injection. Clearly, more aquifer material could be treated if the organisms or the aquifer conditions were altered to reduce adsorption and retardation of bacterial movement.

Table 1 . Relative Activity of Constitutive TCE-Degrading Strains Developed During Phase I

Strain Designation	Parent Strain	Relative Activity (%)*
ENV JG370B	*Pseudomonas* ENV BF1	25
ENV JG370C	"	29
ENV JG370E	"	31
ENV JG370G	"	36
ENV JG370J	"	22
ENV JG371B	"	36
ENV JG371C	"	25
ENV JG371D	"	25
ENV JG445K	"	50
ENV JG445L	"	29
ENV JG445M	"	22
ENV JG446A	"	29
ENV JG443A	"	24
ENV JG446H	"	20
ENV JG458	"	22
ENV JG370A	"	38
ENV JG421	"	19
ENV JG335H	"	19
ENV JG332E	"	19
ENV JG447	*P. cepacia* G4 ENV CM1-9 (adhesion deficient)	27
ENV JG166B	"	17
ENV JG327B	"	19
ENV JG326	"	42
ENV JG360	*Pseudomonas* ENV110	19
ENV JG361A	"	22
ENV JG361B	"	17
ENV JG363A	"	17
P. cepacia $PR1_{301c}$ (Malcom Shields)	*P. cepacia* G4	28

* Activity of lactate grown cells relative to phenol-grown *P. cepacia* G4 as measured by a ^{14}C-toluene assay.

Previous studies have demonstrated the feasibility of generating adhesion-deficient variants of a number of different bacterial strains by transposon mutagenesis, chemical mutagenesis, or by selection of naturally-occurring adhesion-deficient variants from a population (Bayer et al., 1983; Anderson et al., 1988; Gong and Forsberg 1989; Crews et al., 1990). In one study greater than 91% of a highly adhesive strain of *Pseudomonas fluorescens* (Pf0-1) was consistently retained by a 3 cm tall, 12 g sand column (DeFlaun et al., 1990). With this degree of attenuation, *in situ* dispersal would be limited to approximately 0.2 M in an unconsolidated sand aquifer. However, an adhesion-deficient variant (Pf0-5) with 40% retention in the same sand column would be capable of dispersing approximately 1 M in a sandy aquifer.

By using this simple adhesion assay, ENVIROGEN has selected both transposon generated mutants and natural variants of *P. cepacia* G4, *M. trichosporium* OB3b, *P. cepacia* PR1$_{301c}$, *P. mendocina* KR1, and *P. cepacia* ENV BF1 that are adhesion-deficient in comparison to the wild-type. Some of the variants adhere to the sand columns at <10%, while the wild type strains adhere at >90% (Table 2). Model aquifer studies at ENVIROGEN have demonstrated that several of these strains migrate through a 25 cm sand-column at the same rate as a chloride tracer (Table 4).

These metabolically proficient, adhesion-deficient strains are more suitable for *in situ* applications than the wild-type organisms because they are less likely to plug the injection well during application and should travel farther into the subsurface, thereby increasing the effective zone of remediation. Because many of these variants also constitutively degrade TCE, they will continue to degrade as they penetrate contaminated aquifers.

Degradation of TCE Sorbed to Aquifer Solids. Because of the tendency for chlorinated solvents to adsorb tightly to aquifer solids, ENVIROGEN is performing extensive research to evaluate the ability of degradative microorganisms to remove TCE sorbed to solid surfaces. Early work has demonstrated that TCE is rapidly degraded from sand surfaces, and that the rates observed cannot be attributed to desorbtion kinetics alone. It is presumed that the contact of the contaminated particles with hydrophobic surfaces of the degradative bacteria promotes removal of the contaminants, and that the concentration gradients caused by the rapid degradation of the desorbed contaminant further promotes removal. The use of adhesion deficient microorganisms improves the removal of TCE from aquifer solids because of the enhanced contact of the surfaces with the degradative organisms. Work is continuing in this area to confirm mechanisms of desorbtion and to determine environmental factors that affect the process.

Enhancing Energy Reserves in Degradative Organisms. In addition to problems of gene/enzyme induction and adhesion, microbial biocatalysts usually require the addition of nutrients to stimulate and prolong their activity. All of the organisms ENVIROGEN uses to degrade TCE require a co-substrate for sustained enzyme activity and metabolic survival. However, adding a co-substrate to an aquifer can stimulate the growth of other non-TCE-degrading organisms and create problems with biofouling and oxygen depletion. Adding methane to an aquifer is difficult and supports the growth of non-degrading organisms, and the addition of toluene or phenol, the co-substrates ENVIROGEN uses in TCE bioreactors, may reduce water quality. Both phenol and toluene are classified as hazardous substances and are regulated under RCRA, the Clean Water Act and the new Clean Air Act. Furthermore, the use of co-substrates adds to the cost of the treatment process and may be operationally inefficient if the microbes are transported or migrate away from the co-substrate.

Table 2. ENVIROGEN'S adhesion-deficient TCE-degrading microorganisms

Strain Designation	Parent Strain	% Adhesion
P. cepacia G4	Wild Type	>90
ENV 1CB (transposon)	"	20
ENV CM7	*P. cepacia* G4	37
ENV CM1-9	"	3
ENV JG447 (constitutive)	ENV CM1-9	3
ENV JG116B (constitutive)	"	3
ENV JG327B (constitutive)	"	3
ENV JG326 (constitutive)	"	3
P. cepacia $PR1_{301c}$ (constitutive, Shields et al.)	*P. cepacia* G4	>90
ENV PR1-A (constitutive)	*P. cepacia* $PR1_{301c}$	47
ENV PR1-B (constitutive)	"	89
ENV PR1-C (constitutive)	"	64
Pseudomonas ENV BF1	Wild Type	>90
ENV BF1CM1	*Pseudomonas* ENV BF1	89
ENV BF1CM2	"	40
ENV JGB-1	"	60
P. mendocina KR1	Wild Type	>90
ENV E5 CM1	*P. mendocina* KR1	19
ENV E3 CM2	"	4
ENV B CM2	"	8
ENV E CM2	"	10
ENV PM5 (constitutive)	"	<10
ENV PM8 (constitutive)	"	<10
ENV PB5A W16B (const.)	"	26
ENV PB5A W22 (const.)	"	<10
ENV 3052A W22 (const.)	"	<10
ENV 3052A W17B (const.)	"	39

An alternative approach for enhancing and maintaining biological activity *in situ* is to utilize biocatalysts that are enriched in energy reserves. The production of energy storage polymers, most commonly poly-ß-hydroxybutyric acid (PHB), by bacteria is a long-studied phenomenon (Lemoigne, 1926). PHB is produced by many bacteria, under conditions of a nutrient limitation in the presence of excess carbon, and may account for up to 80% of the bacterial cell dry weight. Utilization of PHB as a reducing power substrate by methanotrophic bacteria degrading TCE has been demonstrated (Henry and Grbic'-Galic', 1991; Henrysson and McCarty, 1993).

The primary advantage of using energy-enriched organisms for *in situ* remediation versus feeding indigenous or introduced organisms is that it forces more efficient utilization of added oxygen and inorganic nutrients. The degradative organisms carry an internal food reserve with them into the aquifer. The food reserve is not available to less efficient indigenous organisms, and thus, it does not stimulate increased oxygen demand or biofouling associated with feeding indigenous microbes. The only additional oxygen that may be needed is that required to support oxidation of target contaminants and low rates of cellular respiration.

Unpublished research performed at ENVIROGEN has shown that *P. cepacia* G4 will continuously oxidize TCE for a minimum of 8 hours without feeding cosubstrate after growth in the presence of excess phenol. We have also observed that this organism maintains the TCE degradative enzyme system for a minimum of 12 to 24 hours after the cells are removed from an inducing growth substrate such as phenol. In contrast, another TCE-degrading organism, *Pseudomonas mendocina*, rapidly lost TCE degradative activity if it was not fed a cosubstrate such as toluene (Winter, et al., 1989), but this phenomenon may have been related to rapid enzyme turnover or inefficient culture conditions.

ENVIROGEN has performed extensive research to develop methods for growing organisms so that they contain elevated levels of storage polymers. This research resulted in development of fermentation protocols that allow growth of *P. cepacia* G4, $PR1_{301c}$ and *Pseudomonas* ENV BF1 to optical densities ($O.D._{550}$) of greater than 65 (80 g/L), and so that they contained as great as 60% dry weight of PHB. (Table 3). Only about 5 days are needed to generate the cells, and on going research is demonstrating that this rate of biomass production can be further improved (Figure 8). For example, since the submission of our Phase II proposal, we have grown ENV113 to an cell density of $OD_{550}>80$ while maintaining PHB levels >50%. $PR1_{301c}$ has now been grown to $OD_{550}>75$.

TCE degradation studies with the energy enriched constitutive variants demonstrated that the cells could continuously degrade TCE for up to 165 hr. Non-energy enriched constitutive TOM cells degraded TCE for only about 48 hours. These findings demonstrate that the degradative bacteria can now be cultured so that they have extended TCE degrading capabilities.

ENVIROGEN's ability to generate high density growth of the degradative organisms also provides a significant savings in treatment costs. The high cell densities enable ENVIROGEN to produce significantly more degradative biomass than competitors without increased fermentation costs. Because the cells also contain PHB that allows them to degrade more TCE, the total amount of TCE degraded per fermentation is increased.

Continuing Challenges for *In Situ* Bioremediation

ENVIROGEN has developed an extensive collection of degradative microorganisms for aerobic *in situ* bioremediation of VOC-contaminated aquifers (Tables 1 and 2, Figure 1), and we are approaching commercialization of the technology. Before the technology can be commercialized, however, additional pilot and field testing is needed. Modeling at both the microcosm and field scale will provide insight into the types of contaminated aquifers that may be subjected to treatment by this technology, and the cost of full scale implementation. Because few field studies of bioaugmentation in aquifers have been performed, there is a shortage of information regarding the performance of added microbes *in situ*. Extensive pilot scale modeling with both actual and model aquifer materials will help predict how the microbes will perform *in situ*, and initial field demonstrations will allow testing of the models.

Modeling is needed to predict the effect of aquifer solid composition (grain size, heterogeneity, composition, etc.), ground water characteristics (water chemistry, flow rates etc.) and characteristics of the specific degradative organisms on the effectiveness of this treatment process. Although some modeling has been done and/or is in progress, more extensive modeling will allow better predictability. Once the models are developed, field testing will allow confirmation of the models, and allow for appropriate model improvements.

Another area that requires further investigation is the development of methods to support the activity of the organisms *in situ*. Although the generation of energy enriched organisms has significantly prolonged the effective treatment period of the organisms, *in situ* feeding regimes that would not promote significant competition between the added organisms and indigenous populations could further improve the technology. Inexpensive and selective growth substrates should be selected to target the added organisms to extend their degradative activity.

During Phase II of this work, ENVIROGEN will plan to conduct extensive pilot-scale testing for the development of an *in situ* treatment model for applying its TCE-degrading microorganism to contaminated aquifers. The research will be performed in ENVIROGEN's pilot facility and will involve the use of pilot-scale model aquifers. The aquifers will be filled with contaminated model or actual aquifer material and operated to simulate the conditions of an authentic aquifer. Degradative organisms will be added to the aquifer and their migration, degradative efficiency, and survival will be monitored. Concurrently, individual smaller aquifers will be operated with aquifer materials of various compositions, and similar treatment parameters will be determined.

The data generated during the pilot testing will be used to plan a field demonstration of the treatment technology. The field demonstration will involve the identification of a test site with favorable characteristics (based on the treatment model) to insure successful field testing. The field testing will involve adding degradative organisms to the contaminated aquifer and closely monitoring TCE degradation, migration, and survival of the organisms, utilization of oxygen, and other parameters deemed necessary to evaluate the feasibility of this treatment alternative.

Table 3. Maximum growth and PHB production by ENVIROGEN's TCE-degrading strains.

Strain	Cell Density ($O.D._{550}$)	PHB (% dwt)
P. cepacia G4	> 65	60
P. cepacia $PR1_{301c}$	> 65	60
Pseudomonas ENV BF1	> 65	50
Pseudomonas ENV 110	> 35*	N.D.
P. mendocina KR1	> 30	none

N.D. = not yet determined; * not yet maximized

Table 4. Retardation of adhesion-deficient degradative organism migration by aquifer materials

Organism	Sample	sand/silt/clay (%)	Retardation Coefficient*
P. cepacia G4	Wichita 1	99.3 / 0.7 / 0	0.0**
"	SR 10'-20'	87 / 9 / 3	0.0
"	SR 30'-60'	60 / 2 / 38	0.0
"	SR 70'-140'	80 / 7 / 19.3	0.0
"	Play Sand (fine)	100 / 0 / 0	0.0
"	Ottawa Sand (coarse)	100 / 0 / 0	0.75
P. cepacia ENV1-CB (G4 derivative)	Wichita 1	99.3 / 0.7 / 0	1.0
"	SR 10'-20'	87 / 9 / 3	1.0
"	SR 30'-60'	60 / 2 / 38	1.0
"	SR 70'-140'	80 / 0.7 / 19.3	1.0
"	Play Sand (fine)	100 / 0 / 0	1.0
"	Ottawa Sand (coarse)	100 / 0 / 0	1.0
P. mendocina ENV PB5-A	Wichita 1	99.3 / 0.7 / 0	1.0
"	Wichita 2	84 / 5 / 11	1.0
"	Oyster	100 / 0 / 0 (high iron)	0.73
"	Play Sand (fine)	100 / 0 / 0	1.0
"	Ottawa Sand (coarse)	100 / 0 / 0	1.0

* Retardation coefficient = rate of organism migration / rate of chloride migration

** Retardation coefficient = 0.0; organisms did not penetrate the entire length of the column

2. PHASE II TECHNICAL OBJECTIVES

The overall objective of this work is to develop and demonstrate a cost effective technology that can be used to remediate chlorinated solvent contaminated aquifers *in situ*. The proposed technology involves injecting specialized degradative microorganisms directly into contaminated aquifers with the intention of directly contacting and degrading the contaminating solvent. The organisms developed in Phase I produce degradative enzymes in the absence of toxic inducing substrates. They contain energy reserves to allow prolonged degradative activity. The strains developed also do not adhere to aquifer solids, thereby allowing them to penetrate aquifers for improved contact with contaminants. (Tables 1,2,3).

Phase II objectives will include the development of field deployment models that will allow us to predict the effectiveness of these organisms in a wide variety of aquifers in the field, and to demonstrate the efficiency of the technology in an actual contaminated aquifer. We also have propose to use the experience obtained during Phase II to develop firm cost estimates for using this technology. The specific objectives of the proposed project are described below.

Technical Objective 1. Generate Predictive Models for Deploying Specialized Degradative Microorganisms. Because of the great variety of contaminated aquifers in the United States and the World, it is important that predictive models be developed so we can pre-determine at what sites the developed *in situ* bioremediation technology will be applicable.

During Phase I, we developed or isolated several specialized organisms for potential field application (Tables 1,2,3; Figure 1). The organisms that currently seem most suitable for field demonstration are derivatives of *P. cepacia* G4, *Pseudomonas* ENV BF1 and *Pseudomonas* ENV110 that are adhesion deficient and constitutively express TCE-degrading enzymes. These organisms can also be grown to high cell density and produce high energy storage polymers (Table 3). Another potentially-important organism developed during Phase I is *Pseudomonas* ENV PC5 because its degradative activity is induced by TCE, a phenomenon not previously observed in any known organisms (Figure 2).

The efficient use of these organisms in the field, and the prediction of their effectiveness in a given aquifer, however, requires the development of predictive models that can incorporate conditions of porosity, flow rates, and other physical aquifer parameters as well as characteristics of the microorganisms such as adhesivity, specific degradative activity, oxygen utilization, and longevity of degradative activity, to determine suitability of this treatment technology. The models must also be developed and tested with data collected in laboratory microcosm studies where conditions can be easily altered, and they must be further tested during a field demonstration to determine their ultimate utility.

Some modeling data was collected during Phase I experimentation. For example, several experiments were performed to demonstrate the migration of adhesion deficient and wild-type bacteria through aquifer materials with different grain size distributions (Figures 3 and 4). These results allowed us to calculate preliminary retardation coefficients for some of the test strains, and to compare the values to those obtained with wild-type organisms (Table 4). The results indicated that the specialized organisms developed in Phase I will migrate through aquifer material at about the same rate as a conservative tracer molecule, whereas the wild type organisms did not migrate well through any of the aquifer materials tested.

We also performed experiments to measure dispersion of adhesion deficient bacteria as they migrated through model aquifer material. Our preliminary experiments were performed in horizontal microcosms with a high flow rate. The horizontal dispersion was minimal (<5cm) over a migration distance of either 40 cm or 1m. During Phase II we will perform more of these experiments with different aquifer materials and lower flow rates to generate dispersion coefficients based on flow rates and porosity as required for accurate modeling.

During Phase I, we also performed experiments to determine amounts of TCE that could be degraded by the specialized organisms, and to measure the effect of bacterial storage polymers on prolonged degradation. In early experiments with wild-type organisms, TCE degradation ceased after about 24 hr. in the absence of inducer. If small amounts of inducer were added, degradation resumed and ultimately cells with storage polymers degraded more TCE than those without (Figure 5). If constitutive TCE degraders were grown to contain storage polymers, they degraded more TCE than cells not containing storage polymers, and they continued to degrade TCE for as long as 168 h (Figure 6). It was difficult to extend these experiments for longer periods of time because we had to continue to add TCE and eventually experienced leaking septa in some vials. These organisms are expected to degrade for even longer periods *in situ* because they will not constantly be exposed to high concentrations of TCE.

We performed an initial experiment to compare the survival of the specialized organisms in aquifer material. *P. cepacia* $PR1_{301c}$ and *Pseudomonas* ENV BF1 were added to aquifer materials at different cell concentrations and incubated for a period of 3 weeks. Both organisms survived well for greater than 28 days (Figure 7). These experiments are still in progress. Work by Krumme et al. (1993), demonstrated that both *P. cepacia* G4 and *P. cepacia* PR1 survived for greater than 10 weeks in model aquifers.

Further proof of concept was demonstrated during oxygen utilization studies. We demonstrated that oxygen utilization rates are less with cells that are not fed co-substrates while degrading TCE, than with fed cells (Table 8). When the cells degrade TCE in the absence of added co-substrates, oxygen concentrations in the samples do not become limiting for many hours. Conversely, if cells were supplied with either phenol or lactate as a co-substrate, oxygen was depleted in <2 hours. These data indicate that feeding co-substrates *in situ* could lead to rapid oxygen depletion and cessation of TCE-degrading activity. Furthermore, even greater oxygen consumption could be expected in actual aquifers where indigenous organisms would also utilize the added substrate and further increase oxygen demands. Thus, if the cells only oxidize their PHB storage reserves as an energy supply longer periods of TCE degradation *in situ* can be expected.

During Phase II, we will compare the ability of each of the specialized degradative organisms to destroy TCE in the presence of a variety of aquifer materials. We will examine specific degradation rates under *in situ* conditions, total amounts of TCE degraded, ability of the cells to degrade TCE sorbed to aquifer material, and length of time during which they continue to degrade TCE. These data will be incorporated into the predictive models to estimate treatment times, the number of organisms needed to reach clean up standards, and to model the effect of aquifer material composition on degradation rates and efficiency.

Technical Objective 2. Demonstrate Utility of Specialized Organisms in Remediating TCE-Contaminated Aquifers. One of the most important steps in moving this technology towards commercial application is the demonstration of its utility in a contaminated aquifer. During Phase II, we propose to use the information gathered from laboratory and pilot-scale microcosm studies to develop predictive models for field application, then apply the technology to a field site for demonstration. The modeling information and site characterization will allow us to design a field deployment system and monitoring plan for the field demonstration.

During a Phase II field demonstration, we will investigate methods for monitoring degradative bacteria and *in situ* degradation of TCE. Monitoring of the introduced bacteria will be accomplished by using standard and selective plating and enrichment techniques as well as gene probes. We have developed gene probes for some of the test organisms proposed for use in Phase II, and we have external support to continue probe development (DOE Phase I SBIR). Other probes, such as catabolic probes for *Pseudomonas cepacia* PR1301c, are also available (Dr. Malcolm Shields, personal communication).

During Phase II, we will also test the specificity of our gene probes for detecting the test organisms among a background of indigenous organisms. This will allow us to select the best gene probe for detecting the test organism, and it will allow a determination of numbers of indigenous organisms within the contaminated site that have the genetic potential for degrading chlorinated solvents.

The demonstration will also allow us to identify factors that could effect the application of this technology in the field. We will identify rates at which degradative organisms can be injected into aquifers, radius of influence of the organisms, effects of aquifer heterogeneities on migration and dispersion, oxygen utilization *in situ*, the effect of protozoan grazing on degradation efficiency and the effect of forced flow gradients on bacterial movement *in situ*. The field demonstration, because of its relatively small size, will allow us to test and control potentially important parameters (nutrients, flow rates, oxygen concentrations etc.) that could effect degradation. Measuring the effects of these parameter changes will then allow us to generate models to aid in the design of future *in situ* remediation systems.

Early in Phase II we will perform fermentation scale-up studies (750L scale) so that we can cost effectively grow energy enriched organisms. During Phase I we developed fermentation techniques that allowed growth of the test organisms, *P. cepacia* PR1301c and *Pseudomonas* ENV BF1, to a cell density of $O.D._{550}$> 65 in only 5 days in a 3 L fermentor (Figure 8). The cells grown to high density contained up to 60% of their dry weight as the high energy storage polymer poly-b-hydroxybutyrate (PHB). More recently we have grown ENV311 to densities of $O.D._{550}$> 80 while maintaining PHB levels >50 dwt.

For field applications we will need more cells than can be generated in our small fermentors. We will, therefore, optimize conditions for growing high densities of energy enriched cells in our large pilot-scale fermentor (750 L). Because of the larger scale, we can test bulk fermentation reagents and carbon sources to minimize costs of biomass production. The larger scale allows for more economic production of biomass, but testing is needed to insure that we can "scale-up" based on our Phase I findings.

Field application also dictates that we examine methods for concentrating, storing and shipping highly active cultures. During Phase I we investigated some of these methods, and determined that *P. cepacia* G4 derivatives (PR1301c etc.) store well at room temperature for short periods of time (1 to 2 weeks), can be stored for longer periods of time at -80 ^{o}C, but do not store well at -20 ^{o}C. During Phase II we will expand these studies to determine storage and shipping conditions for all of the test organisms, and we will investigate the maintenance of storage polymers during storage and shipping.

Technical Objective 3. Generate Costing Models to Estimate Remediation Costs Using the Proposed Technology. As with any new technology, cost estimates are difficult without large scale demonstrations where costs are closely monitored and calculated. A Phase II field demonstration will allow us to estimate the costs associated with this technology. It will also allow us to determine and model the amount of time it will take to remediate contaminated aquifers.

During the Phase II field demonstration, we will closely monitor actual costs of *in situ* remediation. We will compute costs of manpower required, biomass production, site characterization and preparation, field deployment of organisms, utilities, additives required to support activity *in situ*, shipping of organisms and monitoring of the organisms and contaminants. The data generated will be used to develop costing models that will allow cost estimation for full-scale implementation of the technology at various sites around the country.

Table 6 demonstrates the results of a preliminary feasibility cost estimate for our *in situ* TCE bioremediation technology at a "hypothetical" test site in New Jersey. We have purposely used conservative numbers for both the pump and treat estimates (volumes needed), the amount of cells needed to treat the TCE, and the amount of cells we can produce in a single fermentation run. That is, we have elected to estimate our treatment costs on the "high side" and pump and treat on the low side to make conservative cost estimates.

The results of these cost estimates suggest a potential overall savings in both treatment costs (up to 90% savings) and treatment times (up to 38 fewer years). Of these costs, decreased treatment times may be the most important in many cases, especially where an owner wishes to sell a property free of environmental liabilities.

Our ability to culture degradative organisms to high cell density creates a significant commercial advantage by lowering the cost of producing organisms for *in situ* bioaugmentation. The production of degradative biomass is a significant cost consideration for the development of a commercially viable treatment strategy. By culturing greater amounts of cells with fewer fermentation runs, we reduce the treatment cost, and the method becomes more commercially viable.

Summary. During Phase I of this work we developed several specialized degradative microorganisms for remediation of solvent-contaminated aquifers. The organisms constitutively degrade chlorinated solvents, and they are adhesion deficient. We also developed methods for culturing the organisms to high cell density, and so that they contain elevated levels of storage polymers. The storage polymers allow them to degrade more TCE than cells without storage polymers.

The goal of our proposed Phase II work is to expand upon the success of our Phase I work by demonstrating the utility of our specialized degradative microorganisms to remediate contaminated aquifers. Phase II will allow us to develop predictive application models, to improve large-scale fermentation capabilities for the test organisms, to investigate field application of the technology, and to generate the data needed to accurately evaluate the cost of the technology on a full scale.

Table 5. Oxygen Consumption by *P. cepacia* G4

Cell/Culture	Oxygen Consumption (nmoles/min./mg protein)
No PHB, No Carbon	9.87
No PHB + TCE	12.0
No PHB + 20 mM lactate	21.9
No PHB + 2 mM Phenol	36.1
20% PHB	24.1
20% PHB + TCE	24.1

Assays performed with phenol-grown cells @ 16°C in a Rank Bros. oxygen electrode; 2.0 mL, $O.D._{550}$=3.0

Table 6. *Preliminary* cost and time estimates for remediation of a hypothetical New Jersey aquifer by using pump-and-treat or in situ bioaugmentation

Pump and Treat with Activated Carbon Trapping of the TCE:

Pump Times

-- Total plume volume approximately 5.1 million ft^3
-- at 30% pore volume = 11 million gallons of water
-- Pump and treat requires 20-500 pore volumes for 99% removal of TCE
-- @ 100 pore volumes = 1100 million gallons
-- @ 50 gpm, pump for 42 years

Carbon Costs

-- Trapping efficiency of carbon at 1 ppm TCE = 5% (5 lb TCE/100 lb carbon)
-- Design Conditions:
  -- < 3 gpm / ft^2 container
  -- 11 min. residence time
  -- 28 lbs carbon / ft^3
  -- 50 gpm @ 0.5- 1 ppm = 210 lb TCE / yr
  -- Therefore -- 210 lb / yr / 5 % capacity = 4200 lb carbon / yr.
  -- Include 25% margin = 5250 lb carbon / yr

Capital Costs

-- Use 3 carbon filters (4 ft diam. x 7 ft height)	= $15,000
-- Pump (1 horse power for 50 ft head water)	= $ 1,000
-- Bag Filter (for pre-filtration of water)	= $ 900
-- Estimated Total Capital	= $ 16,900

Operating Costs

-- Electrical (1700 w x 24 hr x 365 d @ $0.06 /kwh)	= $ 900
-- Carbon regeneration costs etc. (5250 lb @ $3.00/lb)	= $ 15,700
-- Labor (per yr for changing filters etc.)	= $ 3000
-- Estimated Annual Operating Costs	= $ 19,600
Estimated Treatment Costs (20 yrs)	= **$ 408,900**
Estimated Treatment Cost (40 yrs, 1 equipment replacement)	= **$ 817,800**

In Situ Bioremediation:

-- Bottle Assay Studies -- 10^{10} highly active cells degraded 400 µg of TCE in <48 h
-- ENVIROGEN's current fermentation capacity 1.8 x 10^{16} cells per fermentation run (750L)
-- Theoretically degrade > 700g of TCE/Fermentor of cells
-- Treat >360,000 gallons of groundwater @ 500 ppb, or 3.6 million gallons of water @ 50 ppb, per fermentor run.
-- It could require as few as 15-30 fermentor runs of cells to remediate the test aquifer.

Estimated Cost

-- Biomass production (15 applications/yr @ $4500 / application)	= $67,500
-- Transportation and related expenses (New Jersey Sites)	= $3000/yr
-- Annual Labor (adding degradative organisms etc.)	= $6000/yr
Total Estimated Treatment Cost (2 years, 15 applications)	= **$85,000**
Total Estimated Treatment Cost (2 years, 30 applications)	= **$153,000**

Note: Estimates do not include costs imposed on both technologies, monitoring, permit report writing

Table 7. Potential Field Sites for the Phase II In Situ Technology Demonstration.

Site	Location	Relevant Information
Wurtsmith AFB	Oskoda, Michigan	National technology demonstration site managed by University of Michigan.
Dover AFB	Dover, Deleware	Large field projects currently planned or in progress.
Gilbert-Mosely	Wichita, Kansas	Existing site characterization, and site for demonstration of alternative technologies.
Pease AFB	Portsmouth, New Hampshire	Closed base with TCE contaminated sites.
Hanscom AFB	Boston, Massachusetts	Airstripping/reinjection system in place.
Columbus AFB	Columbus, Mississippi	TCE contaminated site.
Brooks AFB	San Antonio, Texas	TCE plume, site information available.
McClellan AFB	Sacramento, California	National test site with TCE and toluene contamination.
Edwards AFB	California	TCE contamination, demonstration of alternative technology planned.
AF Plant 4	Ft. Worth, Texas	Government owned contractor, large TCE plume, possibly high clay content.

Information provided by Ms. Alison Thomas, AL/EQW, Tyndall AFB, Florida.

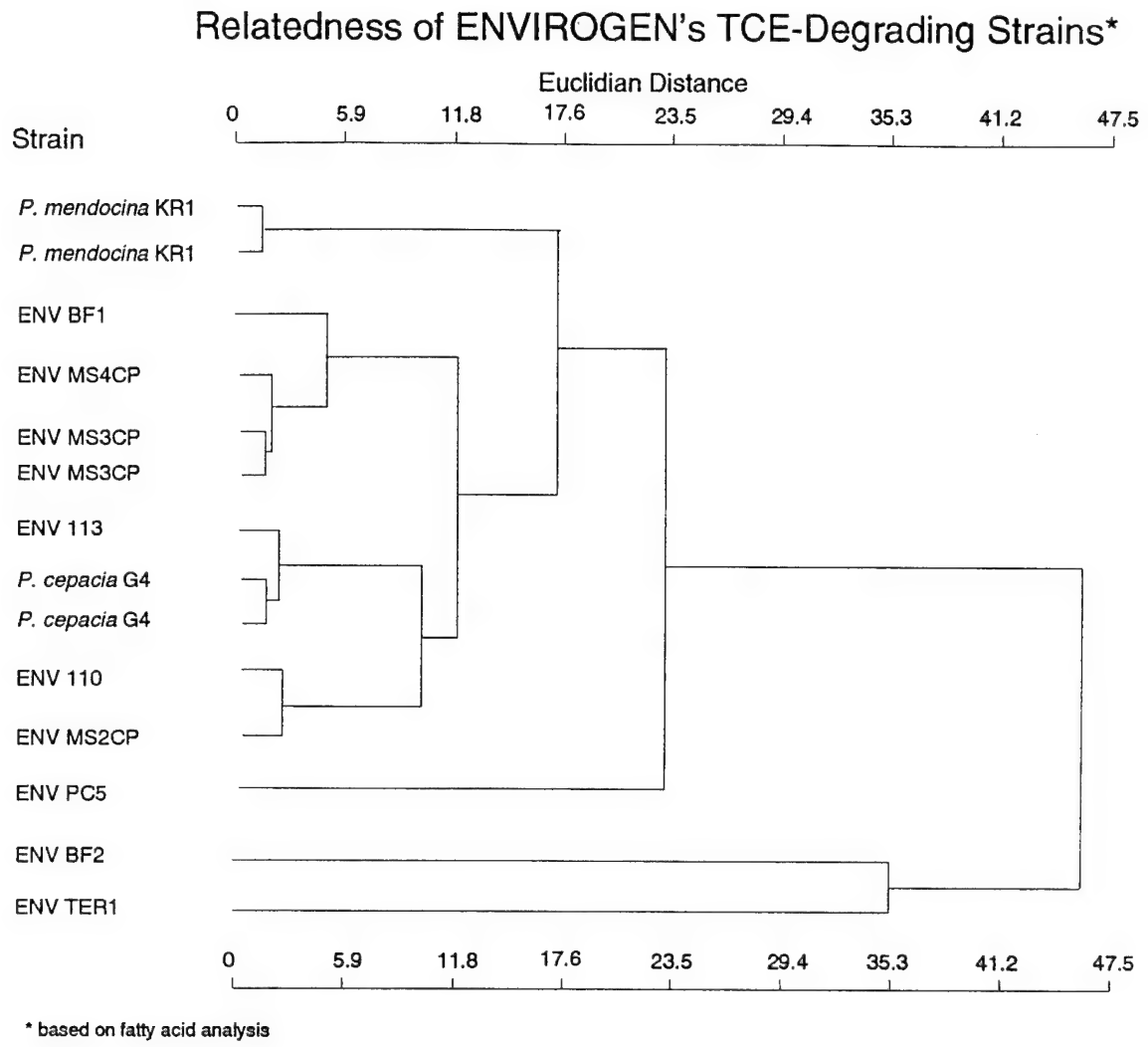

Figure 1. Dendogram of results of fatty acid analysis of ENVIROGEN's TCE degrading bacteria investigated during Phase I. The data were analyzed by using an unweighted pair matching program that compares sample profiles one to another, and then uses cluster analysis to group the strains based on Euclidian Distance. Roughly, samples linked at < 25 are considered of the same species, < 10 same species, < 6 same subspecies or biotype, and < 2 the same strain. We chose to focus our Phase I work on *P. cepacia* G4 (and $PR1_{301c}$), *Pseudomonas* ENV BF1, and *Pseudomonas* sp. ENV110.

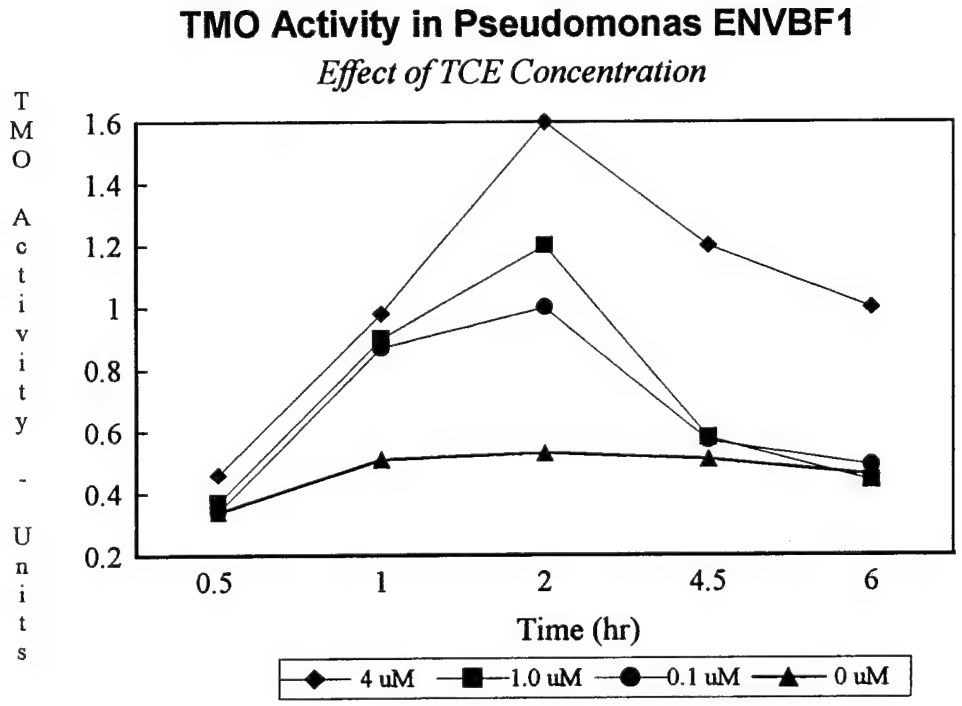

Figure 2. Induction of TCE degradation activity in *Pseudomonas* ENV PC5 by various initial concentrations of TCE. Results were based on the use of a ^{14}C-toluene degradation assay, but the exact toluene degradation mechanism (mono- or dioxygenase etc.) has not yet been determined.

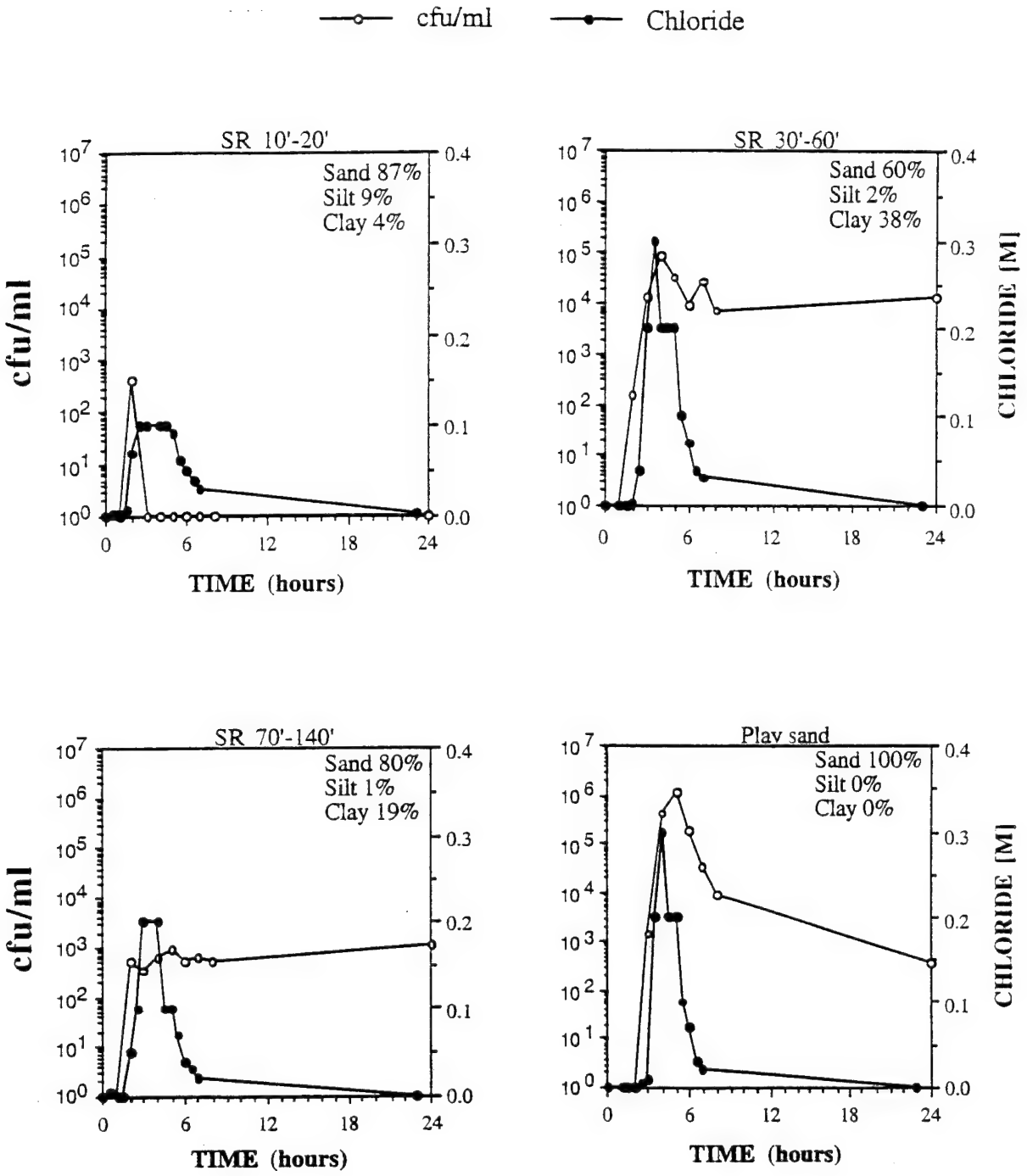

Figure 3. Migration of an adhesion deficient *P. cepacia* G4 through aquifer materials of various grain size distribution. Experiments were performed in 20 x 1 cm glass columns with an up-flow water flow of approximately 8 cm/hr. Aquifer materials were from Savanna River Laboratories.

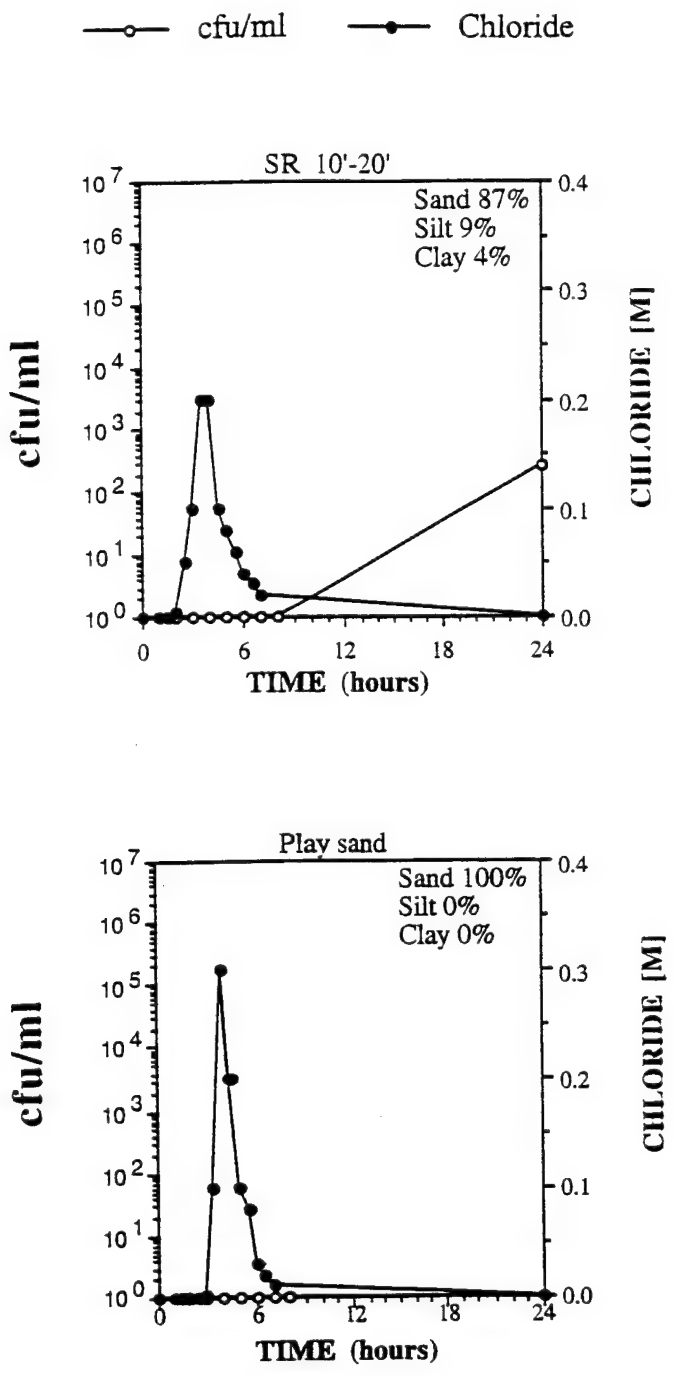

Figure 4. Migration of wild-type *P. cepacia* G4 through aquifer materials of various grain size distribution. Experiments were performed as described for figure 3.

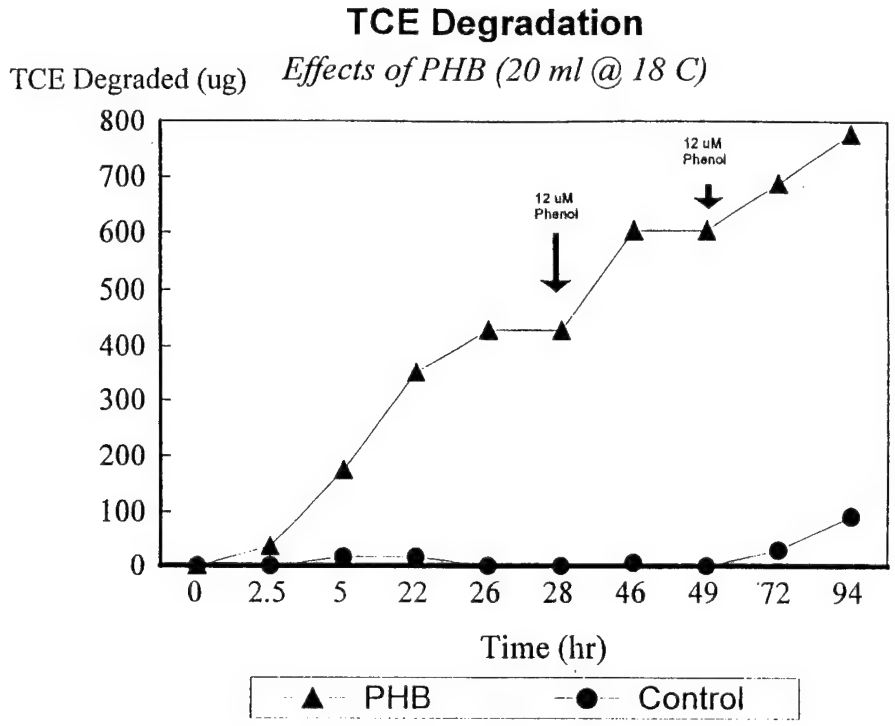

Figure 5. TCE degradation by wild-type *P. cepacia* G4 grown to contain elevated levels of bacterial storage polymers. Low concentrations of phenol were added as an inducer to support prolonged degradation. High specific activity cells were used in this experiment.

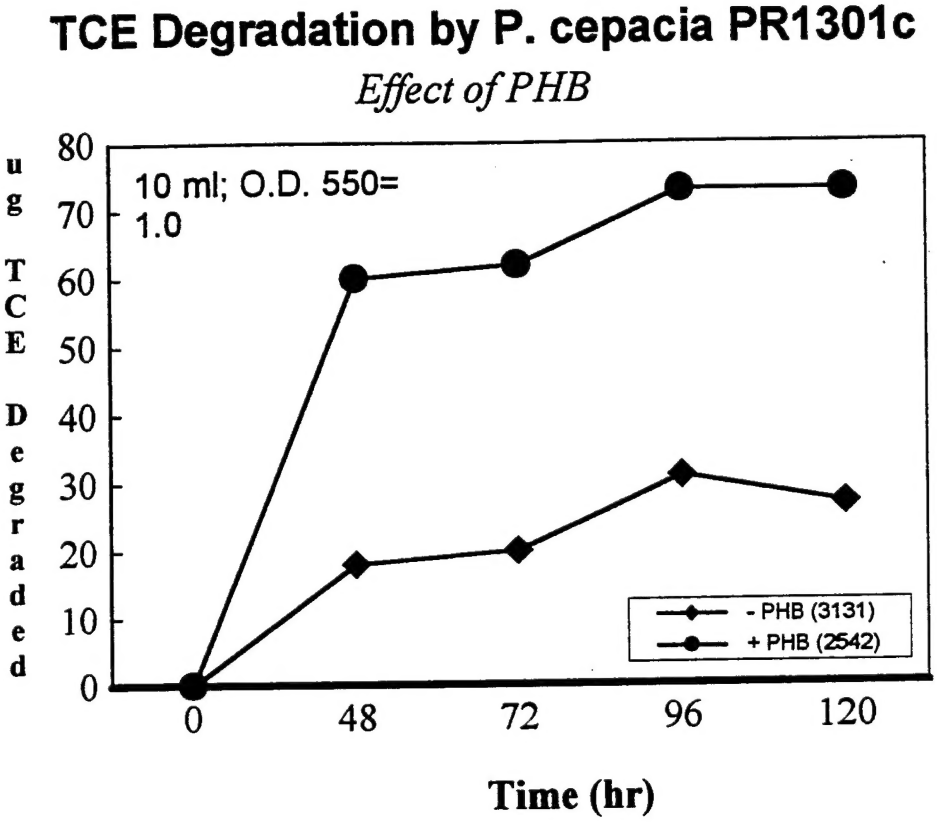

Figure 6. TCE degradation by energy enriched (with PHB) and non-enriched (no PHB) cells of *P. cepacia* $PR1_{301c}$ (constitutive TOM activity). Specific activity as DPM (from ^{14}C toluene assay) is shown in parenthesis. These were low specific activity cells used to minimize the amount of TCE that needed to be added during the time course. Excessive additions of TCE to high specific activity cells (>10,000 DPM) resulted in leaky septa and prevented the extension of time courses.

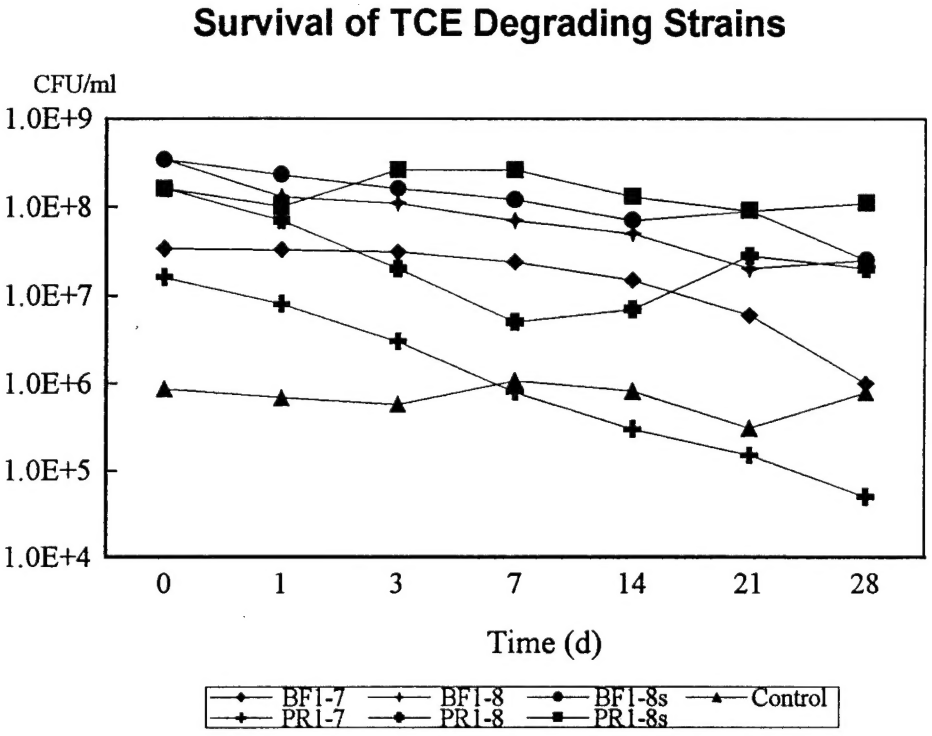

Figure 7. Preliminary study of the survival of *P. cepacia* PR1301c and *Pseudomonas* ENV BF1 in Oyster, VA site aquifer material. Cells were added at either 10^7 or 10^8 CFU/ml of sediment slurry (10g sediment + 5 ml water)

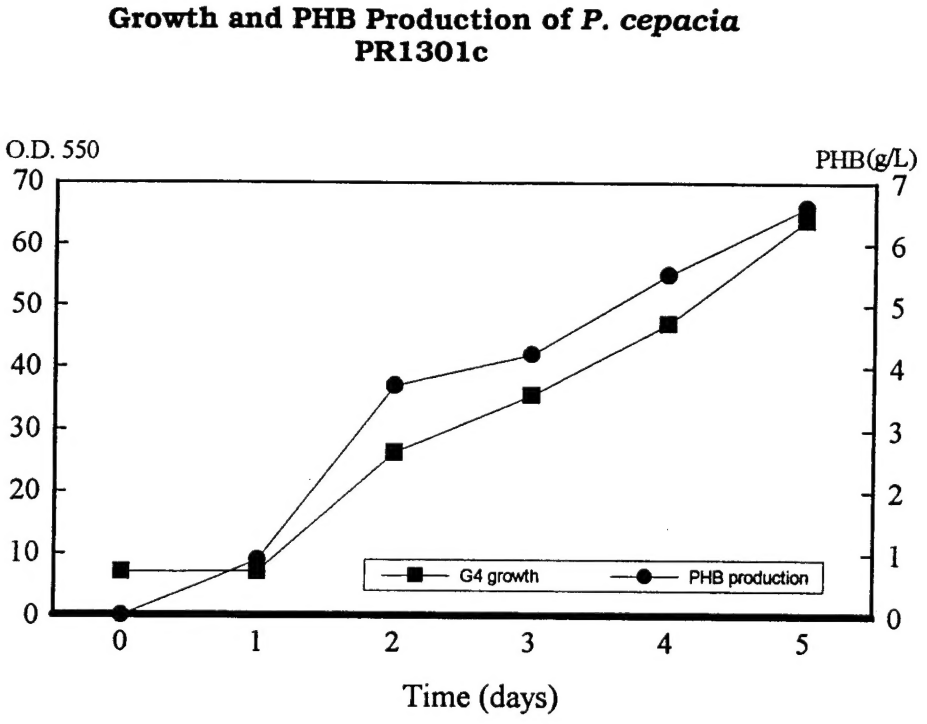

Figure 8. High density growth and PHB production by *P. cepacia* PR1301c in a 3 L fermentor.

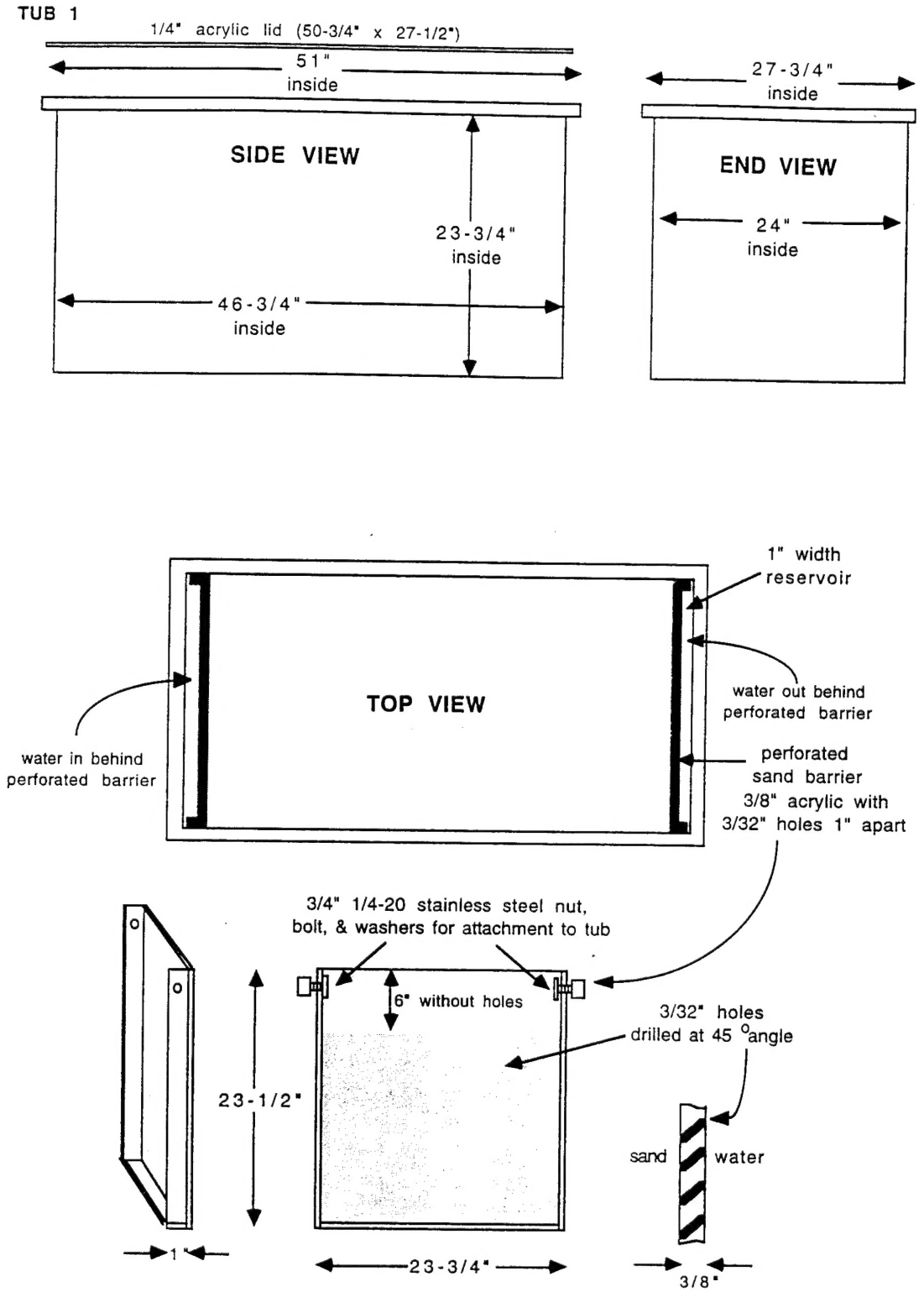

Figure 9. Large-scale model aquifer for use during Phase II pilot testing and model generation.